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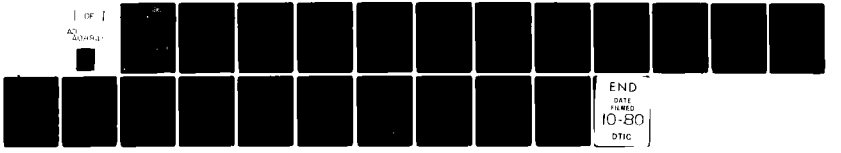

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STUDIES ON PGBx: FRACTIONATION BY A COMBINATION OF DIALYSIS AND MOLECULAR EXCLUSION CHROMATOGRAPHY

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By combining the techniques of dialysis and molecular exclusion chromatography (MEC), a Type III PGBx preparation could be separated into fractions containing different polymer composition and different amounts of in vitro PGBx activity. An over-all purification of PGBx activity was obtained in fraction 3 that contained 20% of the total weight and 40% of the total PGBx activity with a 2 fold purification of specific activity of the PGBx inhibitor factor.
This fraction, which was retained by the membrane on aqueous dialysis, and not retained by the membrane on methanol dialysis, had a long retention time on

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MEC, and gave molecular weight (vapor pressure osmometry) of 1789. Furthermore this fraction was homogeneous by analytical MEC.

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LIST OF ABBREVIATIONS

MEC - Molecular Exclusion Chromatography

HPMEC - High Performance Molecular Exclusion Chromatography

LC - Liquid Chromatography

TLC - Thin Layer Chromatography

AUFS - Absorbance Units Full Scale

VPO - Vapor Pressure Osmometry

S U M M A R Y

By combining the techniques of dialysis and molecular exclusion chromatography (MEC), a Type[1] III PGB_x (1,2) preparation could be separated into fractions containing different polymer composition and different amounts of in vitro PGB_x activity. An over-all purification of PGB_x activity was obtained in fraction 3 that contained 20% of the total weight and 40% of the total PGB_x activity with a 2 fold purification of specific activity of the PGB_x activator factor and only a slight decrease in the specific activity of the PGB_x inhibitor factor.

This fraction, which was retained by the membrane on aqueous dialysis, was not retained by the membrane on methanol dialysis, had a long retention time on MEC, and gave molecular weight (VPO) of 1789. Furthermore, this fraction was homogeneous by analytical MEC.

I N T R O D U C T I O N

The dramatic effect of PGB_x (1,2) in reversing experimentally induced myocardial ischemia and cerebral ischemia in animals was originally reported from this laboratory (3,4). Later, animal studies in other laboratories showed that the administration of PGB_x improved the survival of dogs subjected to lethal cerebral hypoxia (5), or it caused a one hour partial coronary occlusion (6). Because of these results, the use of PGB_x in the possible treatment of similar type human diseases appears reasonable. However, before such human studies may be undertaken it is imperative that the PGB_x preparation be homogeneous, and the active principle identified. Studies in this laboraare underway to separate, purify, and identify the active principle in PGB_x preparations.

PGB_x prepared by the method of Polis et al (1) yields a product that is a hetereogeneous mixture of oligomers of PGB_1. In previous reports, we showed that PGB_x preparations could be spearated into two distinct molecular weight fractions by dialysis against dilute phosphate buffer at pH 6.85 (7) or by gel filtration on Sephadex G-100 (8). When the fractions that were separated by dialysis were analyzed by gel filtration, each fraction showed two components. Thus, the dialysate fraction showed an increase in a more retentive fraction indicating low molecular weight, while the retentate showed an increase in a less retentive fraction or high molecular weight. From these experiments it appeared reasonable to combine the techniques of dialysis and gel filtration in order to fractionate PGB_x into mixtures of narrower molecular weight ranges. This report describes the separation of PGB_x into five fractions with markedly different molecular weight ranges. In addition, these fractions are characterized in terms of in vitro PGB_x activity, UV absorption spectra, and molecular weight.

[1]To avoid confusion, PGB_x Fraction 3 from Sephadex LH-20 MEC (1) is called Type III to distinguish it from "fraction 3" separated in this study. Similarly Type II refers to Fraction 2 from Sephadex LH-20 Chromatography.

EXPERIMENTAL

METHODS

PGB_X acid (Type III), prepared according to Polis et al (1), was used in this study. The PGB_X acid was converted to the sodium salt by dissolving the acid in ethanol, diluting the ethanol to 60% and titrating the ethanolic solution with aqueous 1.0 N NaOH to pH 7.2-7.4. The ethanol was first evaporated off and the remaining solution shell frozen and lyophylized. The in vitro PGB_X nitochondrial effect was assayed according to Polis et al (1) as modified by Shmukler et al (9). UV absorption spectrometry was carried out with a Cary Model 14 spectrophotometer (Varian Instruments, Sunnyvale, CA). Molecular weights of the free acids were measured by vapor pressure osmometry using a Wescan Molecular Weight apparatus (Corona Electric Co., Ltd. Japan). Molecular weights of PGB_X salts were also measured by high performance molecular exclusion chromatography (HPMEC) on Ultrogel AcA54 in 0.05 M phosphate buffer, pH 6.85 containing 0.001 M NaN_3 as a preservative. The column (0.9 cm I.D. x 20.5 cm long) was packed according to the manufacturer's instructions. HPMEC was carried out with a modular chromatograph consisting of: (a) Laboratory Data Control Mini Pump (Riviera Beach, FL); (b) Injector (Altex Inc., Berkely, CA); (c) UV detector set at λ254 nm (Laboratory Data Control); and (d) strip chart recorder (Linear Instruments, Irvine, CA). The chromatographic parameters were : flow-rate, 0.2 ml per minute; column pressure, <10 psi; temperature, room ambient; chart speed, 0.2 cm per minute; and detector sensitivity, 0.16 AUFS. The system was first calibrated with a series of sodium polystyrene sulfonate standards (Pressure Chemical Co., Pittsburgh, PA) of known molecular weight and narrow polydispersity range. Figure 1 shows the retention volume for these standards plotted as a function of the log of their molecular weights. The solid line is the best fitting curve obtained by linear regression analysis of 5 points beyond the void volume (Vo), indicated by the arrow at 5.0 ml. The second arrow indicates the total fluid volume (Vt) of the column. This calibration curve was used to calculate the molecular weight of the sodium PGB_X samples from their retention times. When PGB_X samples exhibited more than one peak, the molecular weights of both peaks were calculated. In addition, from the area under each peak and their corresponding molecular weights, the number average molecular weight ($\bar{M}n$), weight average molecular weight ($\bar{M}w$) and the polydispersity ratio, $\bar{M}w/\bar{M}n$ were calculated.

The Ultrogel packing (LKB Products, Bromma, Sweden) used in this study is less compressible than the Sephadex packing used in a previous study (8). Because of this, the solvent could be delivered with a positive displacement pump under low pressure and this assured a more constant and more reproducible flow-rate than that realized with the gravity feed system used with Sephadex MEC (8). Nevertheless, since the Ultrogel packing is compressible at higher pressures, care was taken not to exceed the flow-rates and back pressure specified by the manufacturer. Because of the possibility of irreversible changes in the packing volume of solvent flow-rate, the column was calibrated daily. For this purpose a mixture of a sodium polystyrene sulfonates, 1600 and 31,000 daltons was used. This binary mixture was found adequate for daily column calibration since the plot of retention volume against the log of the molecular weight was linear (figure 1). Under these operating conditions, the column has been in use over 6 months with less than 5% increase in retention volume for the calibration standards over this time period.

Preparative MEC was carried out with a modular chromatograph consisting of a Milton Roy Mini-pump, a glass column (2.54 cm i.d. x 40 cm long) packed with Ultrogel AcA54, an Altex UV Detector set at λ254 nm and fitted with a 0.05 cm path length flow cell, a potentiometric strip chart recorder (Texas Instruments, Houston, TX) and a fraction collector (Buchler Instruments, Fort Lee, NJ). The chromatographic parameters were: carrier buffer, 0.05 M phosphate pH 6.85 (no added NaN_3); temperature, room ambient; flow-rate, 1.0 ml per minute; detector attenuation, 1.86 AUFS; and fraction collector set to collect 4 minute fractions. Samples were injected on-column by means of the solvent pump. For this purpose the pump intake was connected to a three-way valve on which a glass syringe was mounted on the top port, the solvent reservoir connected to the bottom port, and the column connected to the side port. By rotating the port selector, either sample or solvent could be selected for application to the column. The sample 2 ml or less, was placed in the glass syringe, the pump started, and the port selector rotated to the "syringe-column" position. When the sample level reached the bottom of the syringe, the walls of the syringe were rinsed with 1.0 ml of buffer and the rinsing pumped onto the column. Two rinses were sufficient to ensure quantitative transfer of the sample to the column. Chromatography was then started by rotating the port selector to the "solvent reservoir-column" position.

FRACTIONATION OF PGB_x INTO MOLECULAR WEIGHT GROUPS

1st Step, Aqueous Dialysis: 2.3 g PGB_x, type III (1) was converted to the sodium salt as described under "Methods" and dissolved in 10 ml of 0.05 M phosphate buffer pH 6.85. This solution was dialyzed against 1000 ml of the same buffer using tubing with a nominal molecular weight cut-off of 12,000. Dialysis was carried out at 4° for 24 hours with continual stirring of the external buffer. At the end of this time the dialysate was replaced with fresh buffer and dialysis continued for an additional 24 hours. The dialysates were then combined, acidified to pH 3.0 with dilute perchloric acid and shaken with 300 ml of ethyl acetate. The phases were separated and the aqueous layer extracted two more times with ethyl acetate. The combined ethyl acetate extracts were washed three to four times with water and the water layer discarded. The combined ethyl acetate phases were flash evaporated at 45° and the residue dissolved in ethanol. This fraction was then converted to the sodium salt (yield 0.540 g). The retentate was quantitatively removed from the dialysis tubing and converted to the free acid (yield 1.81 g).

2nd Step, Aqueous Dialysis 8000: 540 mg of dialysate from step 1 was dissolved in 5 ml 0.05 M phosphate buffer pH 7.1 and dialyzed against 100 ml of buffer using dialysis tubing with a nominal molecular weight cut-off of 8000. The dialysis and recovery of fractions was carried out as described under "1st dialysis." The dialysate, designated "Fraction 1" yielded 0.077 g while the retentate designated "Fraction 2" yielded 0.403 g.

3rd Step, Methanol Dialysis 12000: The retentate from the 1st step (1.81 g) was dissolved in 10 ml of methanol and dialyzed against 1000 ml of methanol with one change of solvent after 24 hours. The dialysate and retentate were flash evaporated separately to yield 1.692 g of dialysate and 0.040 g retentate called "Fraction 5."

4th Step, Gel Filtration: The dialysate from the 3rd step was converted to the sodium salt and dissolved in 5 ml of 0.05 M phosphate buffer, pH 6.85 and chromatographed on the preparative AcA54 column at the rate of 350 mg per charge. Two fractions were obtained as shown in figure 2, which is a typical chromatogram of a preparative separation. The first peak was designated "Fraction 4" and the second, "Fraction 3." The contents of the fraction collector tubes comprising these fractions were combined and concentrated by extraction into ethyl acetate at acid pH. The ethyl acetate was flash evaporated and the residue dissolved in ethanol and stored for later analysis. Figure 3 shows the schematic flow sheet for the separation of PGB_x as described above.

PROPERTIES OF PGB_x FRACTIONS

In Vitro PGB_x Activity: All fractions separated in this study were evaluated for PGB_x activity by methods reported previously (1,9). The results are plotted in figure 4a and 4b. Figure 4c is the assay curve for the starting crude PGB_x Type III preparation used in this study. From these assay curves K_a and K_i were calculated (9) and the data listed in table I. The PGB_x activity, both K_a and K_i, was found in each fraction in varying amounts. Thus fraction 1 contained the lowest K_i activities, while fraction 3 contained the highest K_a with little change in K_i from the starting preparation. From the standpoint of purification of PGB_x activity, fraction 3 contained 20% of the total weight and 40% of the total K_a resulting in a 2 fold purification over the starting material. In contrast the inhibitor factor (K_i) only decreased from 1.25 to 1.08.

Table I

The In vitro PGB_x Activity of PGB_x Fractions Separated by Dialysis and Gel Filtration

Fraction	Wt (g)	%	K_a	K_i	Total K_a
PGB_x Type III	2.30		0.48	1.25	1.104
Separated Fractions					
1	0.077	3.35	0.23	0.25	0.018
2	0.403	17.52	0.64	0.67	0.258
3	0.460	20.00	0.95	1.08	0.437
4	1.190	51.74	0.56	1.21	0.666
5	0.040	1.74	0.57	0.61	0.023
Recovery	94%				125%

Molecular Weight of PGB_x Fractions: The molecular weight of each fraction separated in this study is listed in table II. The first line is the molecular weight of the PGB_x free acid measured by vapor pressure osmometry. The next part of the table lists the molecular weight of sodium PGB_x measured by MEC on Ultrogel AcA54 using sodium polystyrene sulfonates as standards for column

calibration. Since a number of PGB_X fractions showed 2 components on analytical gel filtration the molecular weight of each component of the mixture, the percent composition of the misture, the number average molecular weight ($\bar{M}n$), the weight average molecular weight ($\bar{M}w$) and polydispersity ratio ($\bar{M}w/\bar{M}n$) are listed in table II. The data show that the VPO molecular weights of these fracitons range from 718 to 2296. The lowest molecular weight had passed through 8000 d and 12,000 d dialysis tubing during aqueous dialysis, while the highest molecular weight fraction was retained by the dialysis tubing during both aqueous and methanol dialysis. Intermediate separations were realized by gel filtration of the dialysate from methanol filtration. Analytical MEC of these fractions show that fractions 1 and 3 are homogeneous and fractions 2, 4, 5 and the starting preparation contained two chromatographically separable species.

Table II

The Molecular Weight and Polydispersity of PGB_X Fractions

		Separated Fractions				
	Type III	1	2	3	4	5
MW (VPO)	1749	718	1700	1789	2061	2296
HPMEC						
MW_1	21402	--	26195	--	35607	36546
MW_2	3041	2622	1446	2630	2497	2563
%1	10	--	3.5	--	10	17
%2	90	100	96.5	100	90	83
$\bar{M}n$	4961	2622	2333	2630	5713	8335
$\bar{M}w$	11323	2622	11401	2630	22541	27872
$\bar{M}w/\bar{M}n$	2.28		4.89		3.95	3.34

UV absorption spectra: The PGB_X fractions resolved in this study as well as the starting crude PGB_X were analyzed for UV absorption spectra at a concentration of 0.03 mg/ml. The spectra were similar for all fractions in that they showed an absorption maximum at λ243 nm and an absorption shoulder at λ290-310 nm. The absorbance (mg^{-1} cm^{-1}) was calculated for each PGB_X fraction in the above characteristic spectral region and recorded in table III.

Table III

UV Absorbance of PGB_X Fractions

Fraction	$A^{mg/ml}_{\lambda 243}$	$A^{mg/ml}_{\lambda 300}$	A243/A300
Crude PGB_X	28.33	4.00	7.08
1	25.83	5.00	5.17
2	29.07	5.27	5.52
3	32.17	4.33	7.43
4	31.67	4.03	7.86
5	29.77	5.10	5.84

D I S C U S S I O N

The separation of Type III PGB_X described here yields a product that has a higher specific "in vitro activator" activity and a narrower molecular weight range than the starting preparation. Furthermore this purified fraction 3, is almost equivalent to Type II PGB_X from Sephadex LH 20 MEC (1) in terms of specific "activator" and "inhibitor" activity.

The differences in the molecular weight of PGB_X fractions determined by VPO and MEC cannot be explained completely. One source of difference may be the lack of molecular weight standards of proper molecular structure similar to PGB_X. However, with this type of MEC calibrated with polystyrene sulfonates one would expect differences only on the order of 10-20%, rather than the 4 to 10 fold differences shown in table II. The fact that VPO is carried out with the free acid in methanol, and MEC is carried out with the salt form in aqueous media, suggests that the latter method is complicated by aggregation of PGB_X to form larger molecules. Since all the fractions of PGB_X separated in this study were concentrated by conversion of the salt to the free acid and back to the salt form before MEC, suggests that the molecular aggregates are of constant molecular weight rather than a nonspecific aggregation. This is further born out by the fact that fractions 1 and 3 are homogeneous. Attempts to measure the molecular weight of PGB_X salts by VPO measurement in aqueous media were unsuccessful. It is important to note also that the biological effects of PGB_X, both in vivo and in vitro, are carried out in aqueous media and that the specific molecular aggregation of PGB_X may be important in its mode of biological action.

Figure 1:

Polystyrene sulfonate calibration curve for HPMEC on Ultrogel AcA54. Arrows indicate Vo (5.0 ml) and Vt (12.8 ml). Chromatographic parameters: Column dimensions, 0.9 cm I.D. x 20.5 cm long; carrier solvent, 0.05 M phosphate buffer pH 6.85 containing 0.001 M NaN_3; flow rate, 0.2 ml per minute; temperautre, room ambient; column pressure, <10 PSI.

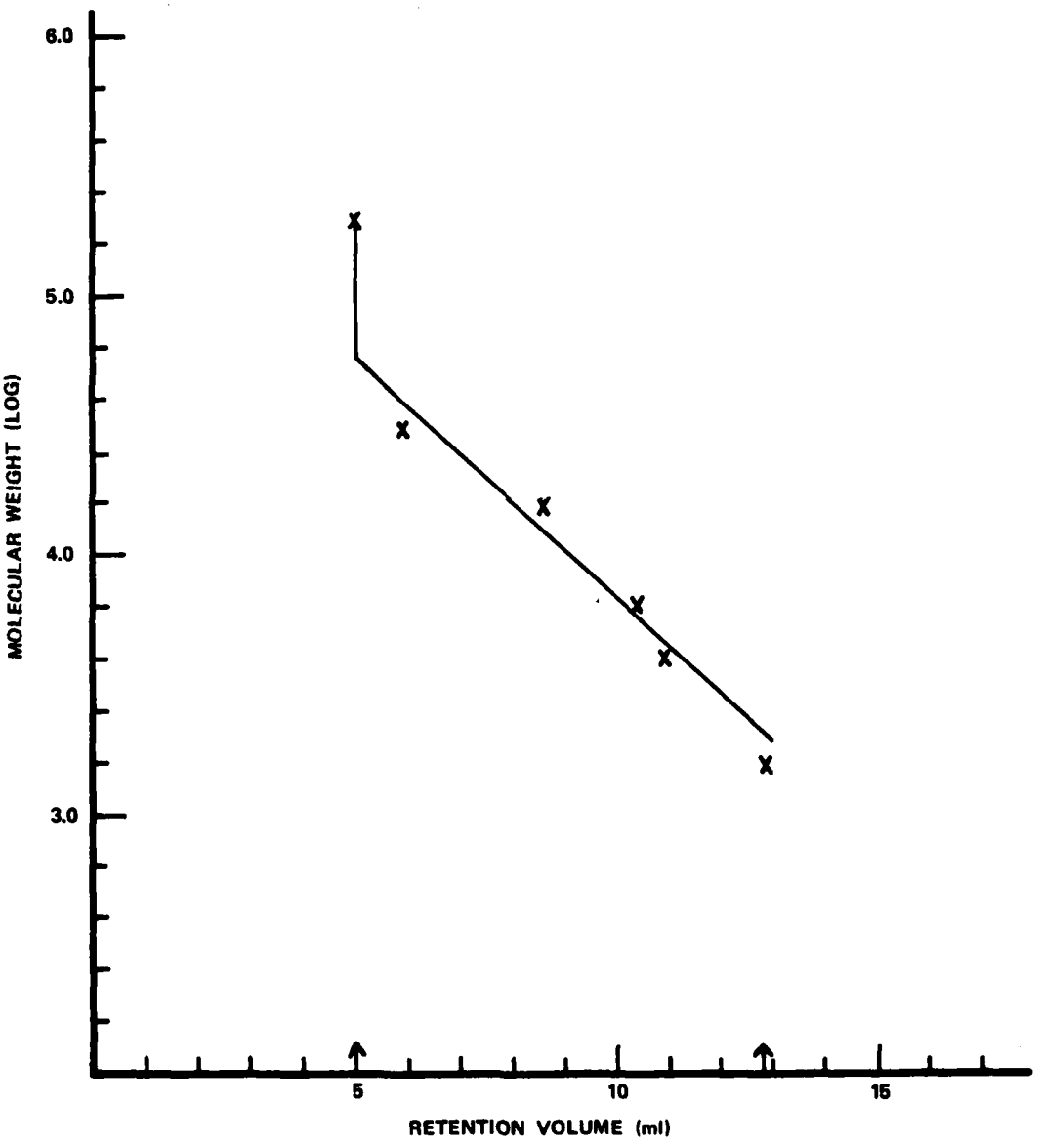

Figure 1 - Polystyrene Sulfonate Calibration Curve for HPMEC on Ultrogel AcA54.

Figure 2:

Preparative MEC of PGB_x Type III on Ultrogel AcA54. Chromatographic parameters: column size, 2.54 cm i.d. x 40 cm long; carrier buffer, 0.05M phosphate buffer pH 6.85; flow-rate, 1.0 ml per minute; fraction collector, 4 minute fractions; detector attenuation, 1.86 AUFS; detector path length, 0.05 cm; temperature, room ambient; maximum PGB_x sample, 250 mg; axis of ordinate, recorder chart scale divisions.

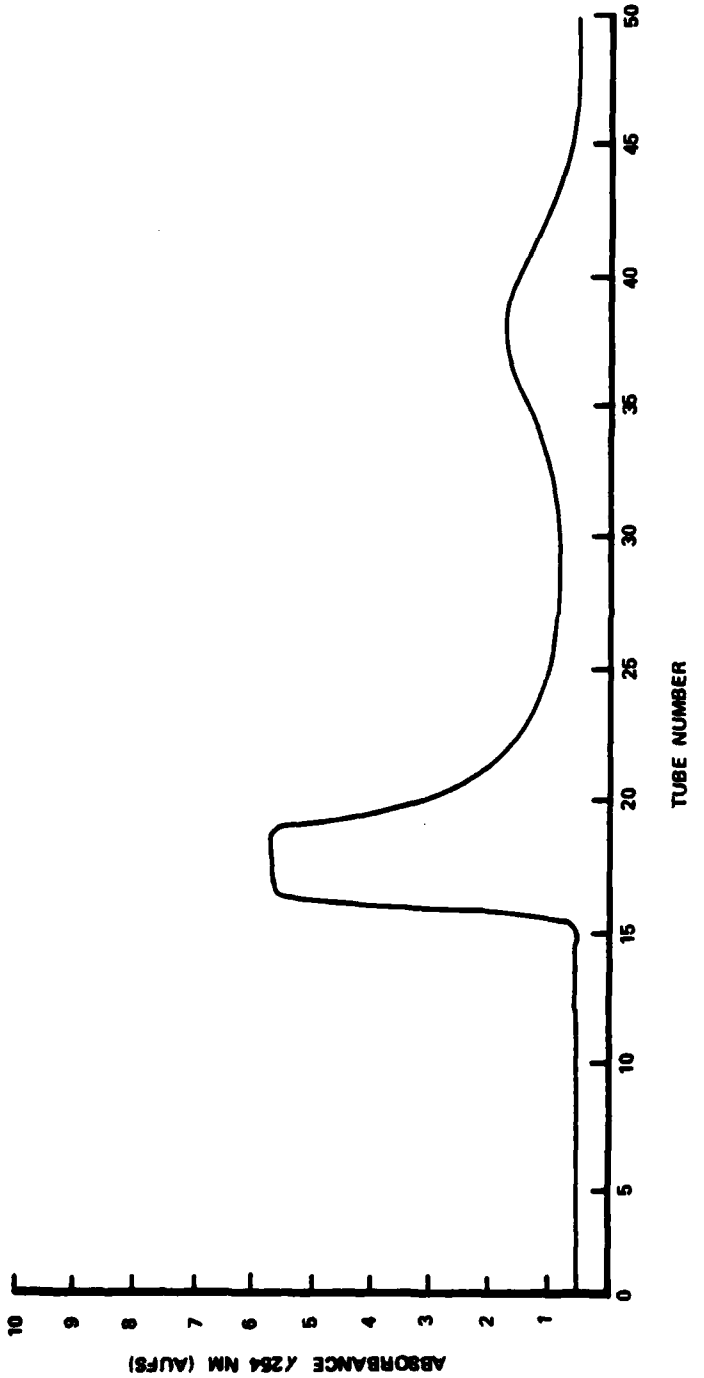

Figure 2 - Preparative MEC of PGB_x Type III on Ultrogel AcA54

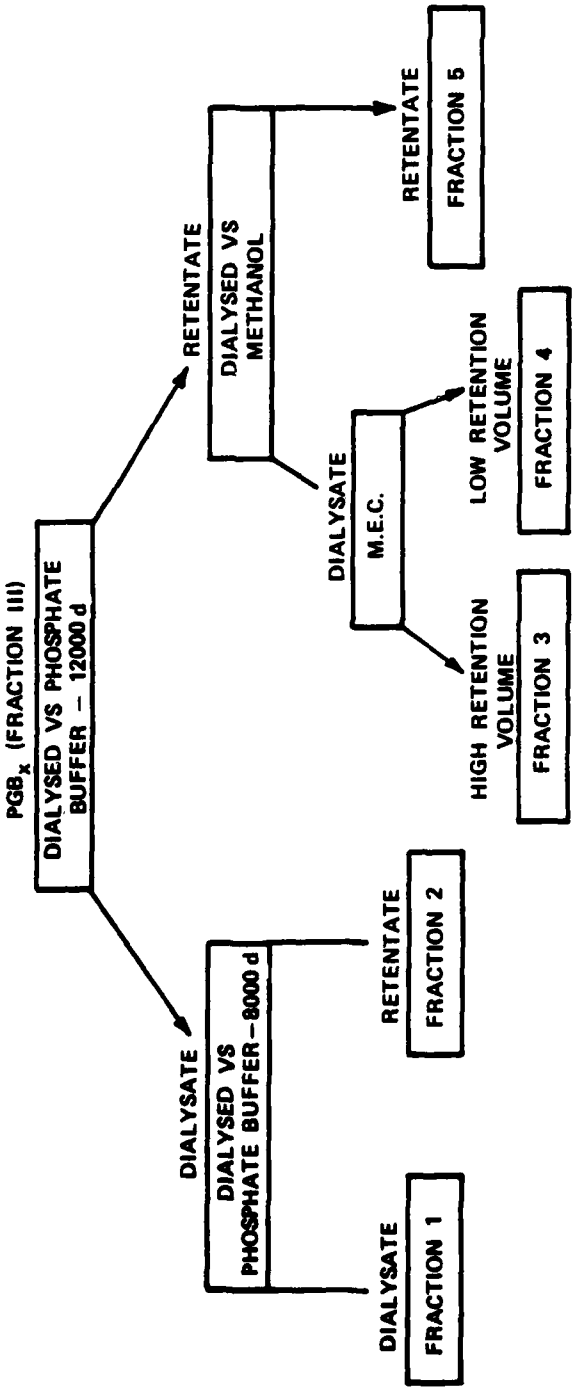

Figure 3 - Schematic Flow-Sheet of Separation Procedure Used in this Study

Figure 4 - Assay Curves for In Vitro Stimulation of Oxidation Phosphorylation of Aged Rat Liver Mitochondria by PGB_X.

Dashed line in all figures refer to standard PGB_X preparation Type II[1]. Numbered curves refer to PGB_X fraction separated in this study. Figure 4a: standard PGB_X, Fraction 1 and Fraction 2. Figure 4b: standard PGB_X, Fraction 3, 4 and 5. Figure 4c: standard PGB_X, dashed curve; PGB_X Type III, solid line.

Reaction medium: Phosphate buffer pH 7.35, 4.98 mM; α-keto-glutarate pH 7.35, 14.93 mM; $MgSO_4$, 4.98 mM; 3-5 day old rat liver mitochondria, 4 mg protein (containing sucrose and EDTA to yield 5.97 mM and 0.010 mM respectively); and water to 2.01 ml. When PGB_X was added, water was reduced an equivalent amount. The mixture was shaken in covered beakers maintained at 27°. At the end of 8 minutes AMP, ADP, KCl and serum albumin were added to a final concentration of 2.27 mM, 2,27 mM, 45.45 mM and 0.68 mg/ml respectively. The reaction was allowed to proceed for 20 minutes and stopped by the addition of 0.5 ml of 31% $HClO_4$. Esterified phosphate was determined as described previously (1).

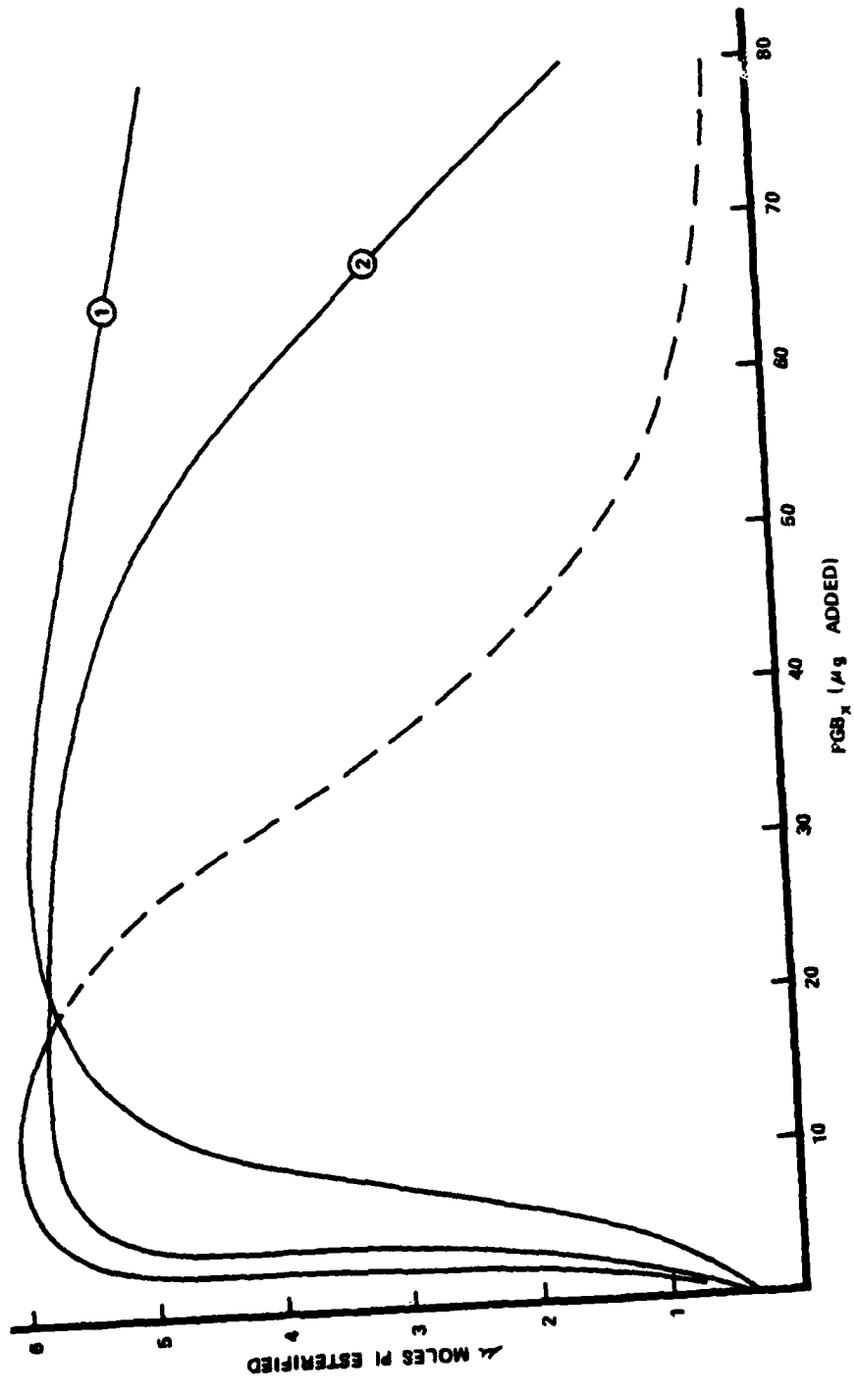

Figure 4a - Standard PGB$_x$, Fraction 1 and Fraction 2

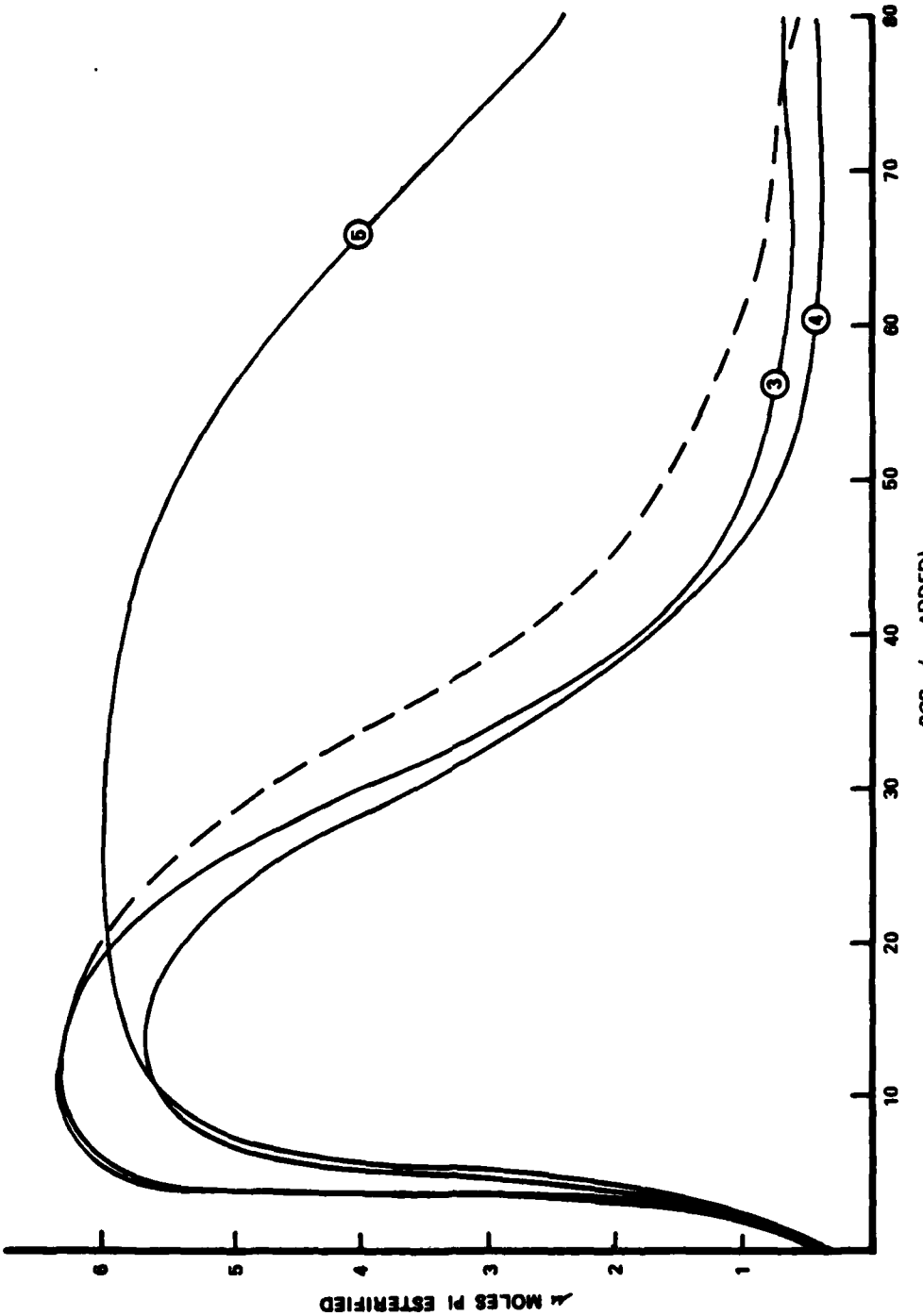

Figure 4b - Standard PGB$_X$, Fraction 3, 4 and 5

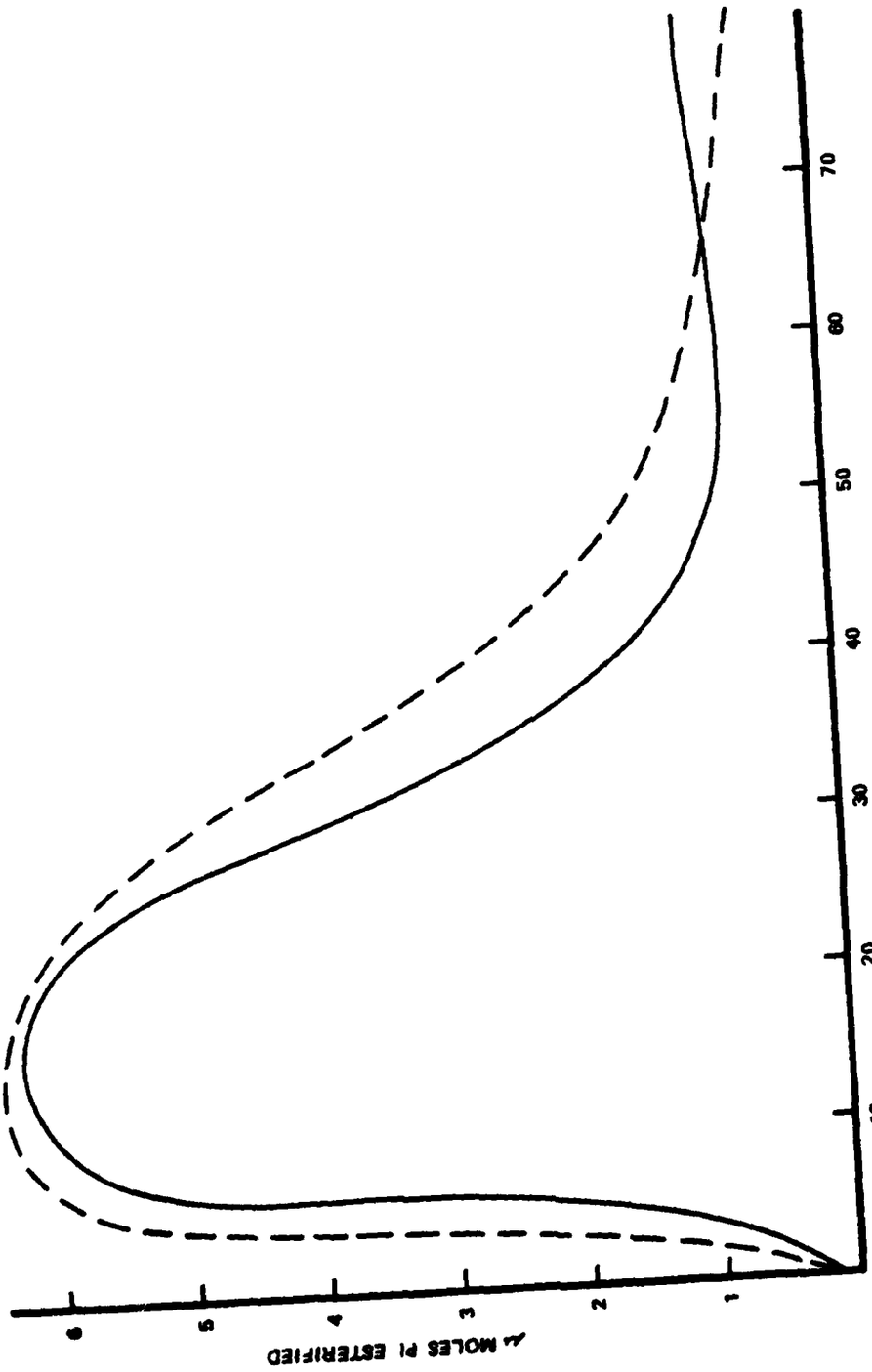

Figure 4c - Standard PGB$_X$, Dashed Curve; PGB$_X$ Type III, Solid Line

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